Ferricytochrome *c* Oxidation of Cobaltocytochrome *c*. Comparison of Experiments with Electron-Transfer Theories†

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ABSTRACT: Electron transfer from cobaltocytochrome *c* to ferricytochrome *c* has been studied by stopped-flow kinetics. The second-order rate constant at pH 7.0, 0.1 ionic strength, 0.2 M phosphate, and 25 °C is 8.3×10^3 M^{-1} s^{-1}. The activation parameters obtained from measurements made between 20 and 50 °C are $\Delta H^{\ddagger} = 2.3$ kcal mol^{-1} and $\Delta S^{\ddagger} = -33$ eu. The rate constant is not significantly dependent on ionic strength; it is also relatively independent of pH between the p*K* values for conformation transitions. The rate diminishes at pH >12. The self-exchange reaction of cobalt cytochrome *c* was investigated with pulsed Fourier transform ^{1}H NMR. The rate is too slow on the ^{1}H NMR scale; it is estimated to be <133 M^{-1} s^{-1}. These results together with the self-exchange rates of iron cytochrome *c* [Gupta, R. K., Koenig, S. H., and Redfield, A. G. (1972), *J. Magn. Reson. 7*, 66] were analyzed by theories of Jortner and Hopfield. The theories predict the self-exchange of ^{Co}cyt *c* to be too slow for ^{1}H NMR determination. The rate constants calculated by the nonadiabatic multiphonon electron-tunneling theory for the ^{Fe}cyt *c*–^{Fe}cyt c^+ and ^{Co}cyt *c*–^{Fe}cyt c^+ electron transfers are in good agreement with experiments.

The electron-transfer processes associated with the oxidative phosphorylation and photosynthesis have been the focal points of research in bioenergetics. The *c*-type cytochromes play key roles in these processes, such as electron transfer between reduced and oxidized cytochrome *c* and between cytochrome *c* and physiological oxidoreductases and the bacteriochlorophyll complex. However, the understanding of mechanisms for electron transfer between these enzymes is shallow. In fact, the study of electron transport between cytochrome enzymes at the molecular level, or for that matter of protein–protein redox reactions in general, is in extreme infancy. Electron transfers between two protein molecules which have been reported are: cyt *c*–cyt c_{551} (Morton et al., 1970), cyt *c*–cyt *c* (Kowalsky, 1965; Gupta et al., 1972; Gupta, 1973; Oldfield and Allerhand, 1973), cardiac cyt c_1–cyt *c* (Yu et al., 1973); cyt c_{551}–cyt c_{551} (Keller et al., 1976), and ^{Co}Hb[1] and ^{Fe}Hb mediated by methylene blue (Dickinson and Chien, 1975a). There have been many investigations on the redox reactions of heme proteins with simple inorganic metal complexes. There have also been elegant studies (Rosenberg et al., 1976; Wherland et al., 1975; Holwerda and Gray, 1975) on the $Fe(EDTA)^{2-}$ reduction of azurin, plastocyanin, stellacyanin, and laccase. Discussions of other redox reactions of hemeproteins with simple reagents have been recently reviewed (Bennett, 1973).

The scarcity of data for protein–protein electron transfers had not thwarted postulation of mechanistic models for the processes. The Winfield mechanism (Takano et al., 1973) proposed elaborate electron pathways for cyt *c*. Transfer via conduction band (De Vault and Chance, 1966) and quantum mechanical tunneling (DeVault and Chance, 1961) has also been invoked. Two other models are of greater interest because of their quantitative aspects. The first is the theory of outer-sphere transfer of Marcus (1963, 1964) most commonly employed in the relative form (Marcus and Sutin, 1974). The second one is the theory of multiphonon nonadiabatic electron tunneling (Hopfield, 1974, 1977; Potasek and Hopfield, 1977; Jortner, 1976). The central purpose of our program is to carefully study electron transfer between protein molecules, to consider the relative merits of various models, and to suggest possible direction for refinement of theories.

We have synthesized cobalt-substituted cytochrome *c* (^{Co}cyt *c*) and found it to be nativelike in its structures, spectra, and physical and biochemical properties (Dickinson and Chien, 1974; Chien et al., 1975; Dickinson and Chien 1975b,c). It has a value of −140 mV for half reduction potential, so its oxidation by ^{Fe}cyt *c* will be driven by an $\Delta E_{m,7}$ of 400 mV. A study of this cross-exchange reaction

$$^{Co}\text{cyt } c + {}^{Fe}\text{cyt } c^+ \xrightarrow{k_{12}} {}^{Co}\text{cyt } c^+ + {}^{Fe}\text{cyt } c \qquad (1)$$

as well as the self-exchange reaction

$$^{Co}\text{cyt } c^* + {}^{Co}\text{cyt } c^+ \xrightarrow{k_{11}} {}^{Co}\text{cyt } c^{+*} + {}^{Co}\text{cyt } c \qquad (2)$$

have been made and the results reported here. Comparisons are made with the third reaction

$$^{Fe}\text{cyt } c^* + {}^{Fe}\text{cyt } c^+ \xrightarrow{k_{22}} {}^{Fe}\text{cyt } c^{+*} + {}^{Fe}\text{cyt } c \qquad (3)$$

Materials and Methods

Materials. Ferricytochrome *c* (Sigma, beef heart type VI) was used without further purification. Cobalt cytochrome *c* was prepared, fractionated, and reduced as previously described (Dickinson and Chien, 1974, 1975b). All solutions, unless otherwise indicated, were prepared in a standard pH 7.0, 0.02 M phosphate buffer containing NaCl to give an ionic strength of 0.1. Solutions at other pH values were prepared with citrate phosphate (pH 3–6), phosphate (pH 7–8), and borate (pH 9–10.5) buffers made up to 0.2 ionic strength. When ionic-strength dependence was studied, it was adjusted with NaCl. All stock solutions were exhaustively freed of

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[1] Abbreviations used are: ^{Co}cyt *c*, cobaltocytochrome *c*; ^{Co}cyt c^+, cobalticytochrome *c*; ^{Fe}cyt *c*, ferrocytochrome *c*; ^{Fe}cyt c^+, ferricytochrome *c*; ^{Co}Hb, cobalt hemoglobin; ^{Fe}Hb, native hemoglobin; $E_{m,7}$, half reduction potential at pH 7.0; ^{1}H NMR, proton nuclear magnetic resonance.

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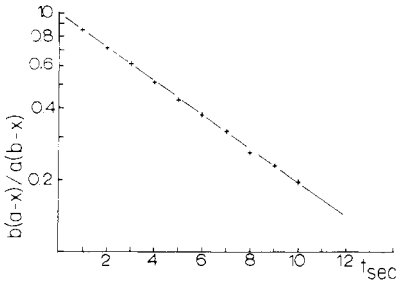

FIGURE 1: Second-order plot for 25 μM ^{Fe}cyt c^+, 9.6 μM ^{Co}cyt c, μ = 0.1, 45 °C, pH 7.0, 0.02 M phosphate solution. The reaction mixture also contained 15.4 μM ^{Co}cyt c^+. The observed wavelength was 4500 Å.

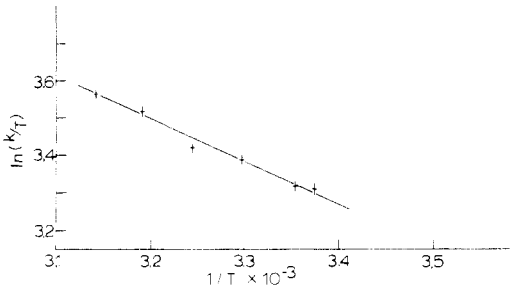

FIGURE 2: Eyring plot of temperature dependence of second-order rates for 25 μM ^{Fe}cyt c^+ and 10.2 μM ^{Co}cyt c over the range 23.2–45 °C, pH 7.0, μ = 0.1, 0.02 M sodium phosphate. The solid line is a least-square fit with slope -1152 ± 130 and intercept 7.18 ± 0.02. k values are the average of separate determinations at 4500 and 5030 Å.

oxygen immediately before use according to published procedure (Dickinson and Chien, 1975b).

Methods. Stopped-flow kinetics were measured with a Gibson-Durrum spectrometer equipped with improved photomultiplier, amplifier, and storage oscilloscope. The ordinate voltages were converted to absorbance value. For a standard ^{Fe}cyt c solution, the calculated absorbance at 550 nm agreed to within 1% of the value obtained with a Cary 14 spectrophotometer. Special precaution was taken to control the temperatures of the stock solutions in the syringe and the mixing chamber to avoid refractive index change artifacts. Baselines were determined with degassed, deionized water at each wavelength used.

Even with rigorous exclusion of air, some autoxidation of the stock ^{Co}cyt c solution occurred over the course of several runs because of its extreme susceptibility to autoxidation. On the other hand, ^{Fe}cyt c is much less readily autoxidized. Only those runs in which the amount of ^{Co}cyt c oxidized was within 1–5% of the amount of ^{Fe}cyt c^+ reduced were reported. In those experiments, the second-order plots were linear over 90% of the reaction. Typically with 25 μM of ^{Fe}cyt c^+ and 15 μM of ^{Co}cyt c the reaction was complete in 30 s with a 25-mV change in voltage and near full scale, virtually noiseless oscilloscope trace. Each data point was repeated at least three times and found to be exactly reproducible. In some experiments there was also a small residual change in absorbance which is never more than 10% of the total absorbance change. This process could not be rationalized as a simple first-order one, as might be expected for a conformation change. Correction by subtracting this change has only a small effect on the rate constant.

The Fourier transform ^{1}H NMR measurements were made on 10 mM solutions of ^{Co}cyt c^+ in 5% D_2O, 0.1 M, pH 7.0, phosphate buffer. One-half milliliter of the solution was introduced into a serum capped and deoxygenated sample tube (Wilmad Royal Crown no. 728). The methyl proton resonance of Met-80 in ^{Co}cyt c^+ is sharp and upfield shifted (Dickinson and Chien, 1975c) as it is in the native ^{Fe}cyt c enzyme. Following a radio frequency pulse saturating this resonance, the magnetization was measured after a sampling time, τ, the range of which is 10–300 ms. The peak height of the recovered signal is designated M_τ. The magnitude of M_∞ was measured directly with an instrumental method which avoided measurements with a very long sampling time; the H_2O resonance was suppressed with a "2-1-4" pulse with a D_2O lock (Redfield et al., 1975). A linear plot of $\ln(1 - M_\tau/M_\infty)$ vs. τ gives a slope of $-T_1^{-1}$.

The spin-lattice relaxation time, T_1, was measured for both fully oxidized ^{Co}cyt c^+ solution and for samples partially reduced using 10–20 μL of 0.1 M sodium dithionite solution, which was calibrated by titrating with a standard 0.1 M $K_3Fe(CN)_6$ solution. Actual extent of reduction was determined by the relative intensity of the Met-80 methyl proton resonance.

TABLE I: Effect of pH on the Rate of ^{Fe}cyt c^+ Oxidation of ^{Co}cyt c.

pH	3.0	4.5	6.0	7.0	7.5	8.0	10.0	12.0
$k_{12} \times 10^{-3}$ [b]	[a]	3.77	3.46	4.25	3.34	3.78	3.78	1.30

[a] Autoxidation dominates. [b] M^{-1} s^{-1}.

Results

The rate of electron transfer from ^{Co}cyt c to ^{Fe}cyt c^+ was monitored at both 450 and 503 nm; the former is the isosbestic point for ^{Co}cyt c and ^{Co}cyt c^+ ($\Delta\epsilon$ = 9.27 mM^{-1}), and the latter for ^{Fe}cyt c and ^{Fe}cyt c^+ ($\Delta\epsilon$ = 2.94 m^{-1}). The data were plotted according to second-order kinetics. Figure 1 shows such a plot for a reaction of 25 μM ^{Fe}cyt c^+ and 9.6 μM ^{Co}cyt c observed at 450 nm. Least-square analysis yielded the second-order rate constant k_{12}. This is averaged with values obtained at 503 nm under identical conditions. At 25 °C, pH 7.0, and 0.1 M ionic strength, $k_{12} = 8.27 \times 10^3$ M^{-1} s^{-1}. There were only a very few cases showing a slight increase in slope of up to 5% at high conversion, which were attributed to errors in measuring small concentrations.

The results obtained from 20 to 50 °C are summarized in the Eyring plot (Figure 2), which gives the activation parameters: $\Delta H^\ddagger = 2.29 \pm 0.25$ kcal mol^{-1} and $\Delta S^\ddagger = -33.0 \pm 0.03$ eu.

Variation of ionic strength from 0.044 to 0.2 has no significant effect on k_{12}. At ionic strengths of 0.044, 0.1, 0.15, and 0.2, the measured values of k_{12} were 8.56×10^3, 8.38×10^3, 7.04×10^3, and 7.70×10^3, respectively. The value of k_{12} is also quite independent of pH from 4.5 to 10 (Table I). However, at pH 3.0 autoxidation of ^{Co}cyt c increases dramatically such that even traces of oxygen make it difficult to determine the electron transfer rate with any precision. Above pH 10 the rate decreases significantly. No reverse transfer of electron from ^{Fe}cyt c to ^{Co}cyt c^+ was expected because of unfavorable thermodynamics; none was observed in several runs to confirm this expectation.

The pulsed Fourier transform ^{1}H NMR spectra were first observed for a fully oxidized ^{Co}cyt c^+ sample from 22.4 to 37.6 °C. The values of T_1 are given in Table II. The same sample was subsequently 41% reduced and the T_1 measured again; the values for these T_1 are also given in the table. The peak height for M_∞ of this partially reduced sample remained constant throughout the experiment, so that its ratio of ^{Co}cyt c/^{Co}cyt

TABLE II: Proton Spin-Lattice Relaxation Times for Cobalt Cytochrome *c*.

Sample		Temp	T_1	f_2
^{Co}cyt c^+ (%)	^{Co}cyt c (%)	(°C)	(ms)	(Hz)
100	0	22.4	266	68670
100	0	33.6	375	
100	0	37.6	482	68702
59	41	23.4	260	68688
59	41	29.1	281	68700
59	41	33.3	427	68715
59	41	38.1	438	
59	41		230	

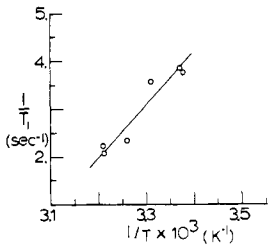

FIGURE 3: Arrhenius plot for data in Table II.

c^+ did not change either. It is noted parenthetically that the resonant frequency, f_2, of the Met-80 methyl resonance shifted with temperature. The observed f_2 at each temperature was noted. Since the chemical shift of the Met-80 methyl resonance is strongly determined by the ring-current field of the porphyrin, the observed variation of f_2 with temperature suggests small changes in the position of Met-80 with respect to the porphyrin ring. Figure 3 is an Arrhenius plot of log T_1^{-1} vs. T^{-1}; the slope gives an activation energy for the spin-lattice relaxation process of 7.6 kcal mol^{-1}.

Discussion

The rate of ^{Fe}cyt c^+ oxidation of ^{Co}cyt c^+ is quite rapid; the rate constant k_{12} for this reaction is 8.3×10^3 M^{-1} s^{-1} (μ = 0.1 M, pH 7, 25 °C). It is comparable to the rate of self-exchange for native cytochrome *c*, the rate constant of which at μ = 0.645 M, pH 7, and 25 °C is 1×10^4 M^{-1} s^{-1} (Gupta et al., 1972). Whereas k_{12} for reaction 1 is essentially independent of ionic strength, k_{22} for reaction 3 is strongly dependent on ionic strength. The value of k_{22} at μ = 0.1 M is only 3.48×10^2 M^{-1} s^{-1}; and the plot of k_{22} vs. $\sqrt{\mu}$ is linear from very low ionic strength to μ = 0.645 M. This dependence is usually interpreted (Frost and Pearson, 1961) as the effect of ionic strength on reactions between charged molecules according to the Debye-Hückel theory,

$$\ln k^\mu = \ln k^\infty - \frac{\overline{Z}_a^2 \alpha \sqrt{\mu}}{1 + \beta r_a \sqrt{\mu}} - \frac{\overline{Z}_b^2 \alpha \sqrt{\mu}}{1 + \beta r_b \sqrt{\mu}} + \frac{(\overline{Z}_a + \overline{Z}_b)^2 \alpha \sqrt{\mu}}{1 + \beta r^{\ddagger} \sqrt{\mu}} \quad (4)$$

where k^μ and k^∞ are the rate constants at μ and infinite ionic strength, respectively, $\overline{Z}_a$ ($\overline{Z}_b$) is the effective active-site charge of molecule a (b), r_a (r_b) is the distance of closest approach, and the two constants are α = 0.509 (2.303) and β = 0.329 $\sqrt{\mu}$ Å^{-1} (water, 25 °C). When $r_a = r_b$ and $\beta r \sqrt{\mu} \ll 1$, one obtains the simplified relationship

$$\ln k^\mu = \ln k^\infty + 2\overline{Z}_a \overline{Z}_b \alpha \sqrt{\mu} \quad (5)$$

Though eq 5 is based on the dilute electrolyte solution theory of Debye and Hückel, it has been observed to be valid for many reactions. When the plot of ln k vs. $\sqrt{\mu}$ is linear, one can estimate a slope the magnitude of $\overline{Z}$. The value of $\overline{Z}$ for native cytochrome *c* thus obtained from the data on self-exchange reaction 3 (Gupta et al., 1972) is +1.3; it is +1.7 from the μ dependence of the rate of reduction of ^{Fe}cyt c^+ by Fe(EDTA)$^{2-}$ (Hodges et al., 1974). Native cytochrome *c* has an overall positive charge of +7.5 and +6.5 in neutral solution (Margoliash and Schejter, 1966) for ^{Fe}cyt c^+ and ^{Fe}cyt *c*, respectively. Therefore, the effective "active-site charge" estimated from eq 5 is much smaller than the total charges. A similar difference between effective and overall charges had been observed for the ionization of methemoglobin (George and Hanania, 1952, 1957). A corollary to this is that the area of exchange site for a protein molecule would be also much smaller than the total surface area of the molecule. Gupta et al. (1972) estimated that for native cytochrome *c* the area of the exchange site corresponds to a circle of 1.5 Å in radius. Other calculations by Wherland and Gray (1976) suggested an effective "active-site radius" of ca. 2 Å.

Unlike the self-exchange reaction 3, the cross-exchange reaction 1 has rates quite independent of ionic strength, suggesting that the "active-site charge" is zero for ^{Co}cyt *c* and that it does not have exactly the conformation of the native species. Subtle structural changes have been seen by ^{1}H NMR in ^{Co}cyt c^+ (Dickinson and Chien, 1975c). Further ^{1}H NMR studies (Chien et al., 1977) showed there is a change in the conformation of ^{Co}cyt c^+ compared to ^{Fe}cyt *c* which is local in nature. The Co-S bond length and/or bond angle changed, which pulled the peptide chain segment 78-82 closer to the heme. There also appears to be a small change in the conformation of a surface residue Tyr-74. Even greater conformational change may occur for ^{Co}cyt *c*, which has an additional electron in the antibonding $3d_z^2$ orbital (vide infra). Therefore, it is conceivable that the portion of the ^{Co}cyt *c* surface which comes into contact for electron transfer is shifted. A shift of only 1-2 Å is probably sufficient to cause the observed independence of k_{12} on μ in view of the small effective area of the exchange site for ^{Fe}cyt *c* (vide supra).

The cross-exchange reaction has only a small $\Delta H^{\ddagger}$ value of 2.3 kcal mol^{-1} but a large $\Delta S^{\ddagger} = -33$ eu. Values of $\Delta H^{\ddagger}$ between 2 to 3 kcal mol^{-1} and ΔS values of −20 to −40 eu have been observed for other redox reactions with protein when there is small or no dependence of the rate constants on ionic strength (Wherland et al., 1975; Rosenberg et al., 1976). The self-exchange process for ^{Fe}cyt *c* has an activation energy of 13 kcal mol^{-1} at low ionic strength ($\simeq$ 0.1 M) and 7 kcal mol^{-1} at high ionic strength ($\simeq$ 1 M) (Gupta, 1973). Part of this activation is said to be associated with the work required to bring two similarly charged reactants together. The 7 kcal mol^{-1} of activation energy at high ionic strength suggests the possibility that activation processes are necessary to change the ^{Fe}cyt *c* and ^{Fe}cyt c^+ molecules into a similar conformation during electron transfer (Cotton and Wilkinson, 1966).

The self-exchange reaction 2 of cobalt cytochrome *c* was studied by the ^{1}H NMR relaxation time of the ring-current-shifted Met-80 methyl resonance of the diamagnetic ^{Co}cyt c^+ molecule. The corresponding resonance in the paramagnetic ^{Co}cyt *c* molecule would be greatly shifted by contact and pseudocontact interactions; its chemical shift has been estimated to be about 14-16 ppm (Dickinson and Chien, 1975c). However, no resonance was observed in this region for ^{Co}cyt *c* probably because of long electron spin-lattice relaxation time. Consequently, if the electron exchange rate between ^{Co}cyt *c* and ^{Co}cyt c^+ is faster than the proton spin-lattice relaxation rate, there will be a decrease of T_1 for the Met-80

methyl resonance according to well-known principles of nuclear magnetic resonance. Examination of the T_1 values in Table II showed that they are the same for samples of fully oxidized and half-oxidized cobalt cytochrome *c*. Therefore, the self-exchange rate is slower than the proton spin–lattice relaxation rate. Though this is a negative result, it does provide an estimate of upper limit for reaction 2. The rate of this process can be described by

$$-\mathrm{d}[^{Co}\mathrm{cyt}\ c^+]/\mathrm{d}t = -\mathrm{d}[^{Co}\mathrm{cyt}\ c]/\mathrm{d}t = k_{11}[^{Co}\mathrm{cyt}\ c^+][^{Co}\mathrm{cyt}\ c] \quad (6)$$

and

$$\mathrm{d}[^{Co}\mathrm{cyt}\ c^+]/\mathrm{d}t = -[^{Co}\mathrm{cyt}\ c^+]/\tau \quad (7)$$

Combining these two equations, one obtains,

$$k_{11} = \frac{1}{\tau[^{Co}\mathrm{cyt}\ c]} \quad (8)$$

From the spread of the pulsed ^{1}H NMR experimental data and taking the largest decrease of T_1 by half-reduction of cobalt cytochrome *c*, we obtain $\tau < 5$ s and therefore $k_{11} < 133\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$.

The relative facile electron transfer for reactions 1 and 3 and very slow electron-exchange rate for reaction 2 can be quantitatively accounted for by current theories. Hopfield (1974) has proposed a semiclassical theory of electron transfer via vibronically coupled electron tunneling. Jortner (1976) formulated a nonadiabatic multiphonon electron-transfer theory incorporating the effect of both low-frequency medium modes and high-frequency molecular modes. Using similar parameters, the two theories can reproduce the observed electron-transfer rates in the light-induced oxidation of cytochrome in photosynthetic bacterium *Chromatium* (Chance and Nishimura, 1960; Olson and Chance, 1960; Morita et al., 1964; DeVault and Chance, 1961; DeVault et al., 1967). The two theories in fact become the same at the high-temperature limit (Jortner, 1976). The theories, originally formulated for electron transfer between molecules fixed in matrix, can be extended to those in solutions (Hopfield, 1977). The biomolecular rate constant for electron transfer between molecules a and b is

$$k_{ab} = 6.023 \times 10^{-4} \left(\frac{2\pi}{\hbar}\right) |\ T_{ab}(r)|^2 (4\pi k_B T\Delta)^{-1/2} \times (2\pi\lambda^3 r/R_p) \exp\left[-(E_a - E_b - \Delta)^2/4k_B T\Delta\right] \quad (9)$$

with the activation parameters

$$\Delta H^{\ddagger} = (E_a - E_b - \Delta)^2/4\Delta - 3RT/2 \quad (10)$$

and

$$\Delta S^{\ddagger} = R \ln\left[\left(\frac{2.38 \times 10^{-2}}{k_B T}\right) \times \left(\frac{2\pi\lambda^3 r}{R_p}\right) (4\pi k_B T\Delta)^{-1/2} |T_{ab}(r)|^2\right] - 3R/2 \quad (11)$$

In the above equations, when the electron is localized at molecule a (b) the nuclear coordinate is q_a (q_b) with k_a (k_b) as the curvature for the wave function and

$$\Delta = k_a q_a^2/2 + k_b q_b^2/2 \quad (12)$$

is the vibronic coupling parameter. E_a (E_b) is the half reduction potential of a (b), k_B is the Boltzmann's constant, T is the temperature in degree Kelvin, r_a (r_b) is the distance of closest approach of a (b) and r is the sum of r_a and r_b. The tunneling matrix element is approximated to be,

$$T_{ab}(r) \approx \frac{2.7}{(N_a N_b)_{1/2}} \exp(-0.72r) \quad (13)$$

where N_a (N_b) is the number of π electrons in a (b). Finally, λ is the characteristic decay constant defined as $\frac{1}{2}(0.72)^{-1} = 0.7$ Å.

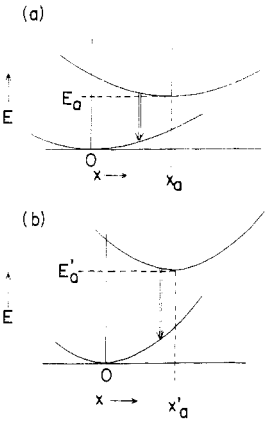

FIGURE 4: The description of an electron-removal process by a configuration-coordinate diagram. The two curves in each figure represent the total energy of the system as a function of the coordinate for the reduced and oxidized states with and without the electron. The schematic diagrams are for (a) ^{Fe}cyt *c* and ^{Fe}cyt c^+; (b) ^{Co}cyt *c* and ^{Fe}cyt c^+.

The half reduction potential and number of π electrons are known for a given molecule and the distance of closest approach is given by x-ray structure. The determination of the vibronic coupling parameter Δ has been discussed by Jortner (1976), Hopfield (1974), and Potasek and Hopfield (1977). Chien (1978) showed that Δ can be calculated from experimental $\Delta H^{\ddagger}$ with eq 10. The quantity Δ is taken to be the sum of Δ_a and Δ_b, which has the value of 0.5 eV for iron-containing molecules and 1.0 eV for cobalt-containing molecules.

The multiphonon nonadiabatic electron-tunneling theory offers for the first time ready calculation of rate constant, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ for electron-transfer reactions involving biological molecules in solution and is exploited here. For the self-exchange of native cytochrome *c*, we note that the heme edges are about 2 Å from the surface according to Dickerson's x-ray structure; therefore, $r = 4$ Å. Together with $\Delta = 1$ eV, one obtains from eq 9–11 calculated values of $k_{22} = 1.7 \times 10^4\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$, $\Delta H^{\ddagger} = 4.9$ kcal mol^{-1}, and $\Delta S^{\ddagger} = -23$ eu. These are to be compared with experimental values at high ionic strength: $k_{22} = 1 \times 10^4\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$, $\Delta H^{\ddagger} = 7.0$ kcal mol^{-1}, and $\Delta S^{\ddagger} = -18$ eu.

To apply the same calculation to the cross-exchange reaction 1, we are guided by physical and chemical intuition to make certain adjustment of parameters. Firstly, we note the absence of ionic strength effect on k_{12}, whereas k_{22} is strongly dependent on μ. This suggests that the contact area for ^{Co}cyt *c* is more remote from the heme edge as it is in the native protein of the order of the effective contact radius (vide supra). For trial calculation, r for reaction 1 is taken to be $2 + 4 = 6$ Å. An adjustment of the vibronic coupling parameter seems necessary as well. Hopfield had assumed a symmetric model in which ϕ_a and ϕ_b have the same configuration-coordinate curves and that $0.5k_a x_a^2 = 0.5k_b x_b^2 = 0.5$ eV (Figure 4). The native cytochrome *c* is low-spin $3d^5$ and $3d^6$ in the oxidized and reduced state, respectively. In the case of cobaltocytochrome *c*, the strongly antibonding $3d_{z^2}$ orbital is populated in the low-spin $3d^7$ configuration. Its effect on the redox potential of ^{Co}cyt *c*

(Dickinson and Chien, 1975b) and on the triggering of allosteric transition in $^{Co}HbO_2$ (Chien and Dickinson, 1973) have already been discussed. The configuration-coordinate curve of ^{Co}cyt c with the antibonding electron should have a greater curvature (Figure 4); i.e., the nuclear motion is made more difficult by the repulsion of the axial ligands. For a trial calculation, we take $0.5k_a x_a^2$ to be 1 eV for ^{Co}cyt c while keeping the value for ^{Fe}cyt c^+ at 0.5 eV.

Substituting the values of $r = 6$ Å, $\Delta = 1.5$ eV, and $E_a - E_b = 0.4$ eV into eq 9-11, one obtains for the cross-exchange reaction $k_{12} = 9.6 \times 10^3$ M^{-1} s^{-1}, $\Delta H^{\ddagger}_{12} = 3.7$ kcal mol^{-1}, and $\Delta S^{\ddagger}_{12} = -21$ eu. These are to be compared with the experimental results of $k_{12} = 8.3 \times 10^3$ M^{-1} s^{-1}, $\Delta H^{\ddagger}_{12} = 2.3$ kcal mol^{-1}, and $\Delta S^{\ddagger}_{12} = -33$ eu. Similarly, we calculated for the self-exchange reaction 2 of cobalt cytochrome c: $k_{11} = 5.1 \times 10^{-3}$ M^{-1} s^{-1}, $\Delta H^{\ddagger}_{11} = 10.6$ kcal mol^{-1}, and $\Delta S^{\ddagger}_{11} = -33$ eu.

In addition to the present study, we have found (Chien, 1978) good agreements between theory and experiments for the following reactions: forward and reverse electron transfer between ^{Fe}cyt c and *Pseudomonas* cyt c_{551}, reduction of ^{Fe}cyt c^+ by Fe(EDTA)$^{2-}$ oxidation of ^{Co}cyt c by Fe(EDTA)$^-$, oxidations of ^{Fe}cyt c by Co(phen)$_3^{3+}$ and by Fe(CN)$_6^{3+}$, autoxidations of ^{Fe}cyt c and ^{Co}cyt c by dioxygen, reversible redox reactions of ^{Fe}cyt c_2 and Fe(CN)$_6^{3+}$, oxidation of bovine cardiac cytochrome c_1 by Fe(CN)$_6^{3-}$ and its reversible electron transfer with ^{Fe}cyt c, phenazine methosulfate catalyzed reduction of methemoglobin by ^{Co}cyt c, reactions of reduced cytochrome oxidase with dioxygen, and the reduction of ^{Fe}cyt c^+ and of ^{Co}cyt c^+ by dithionite and the monomeric $SO_2^-\cdot$. The agreement of rate constants is better than a factor of two for all but two of the reactions where the discrepancies are within a factor of four. The theoretical values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are also in excellent agreement with experimental results, usually within 2 kcal mol^{-1} and 8 eu, respectively. It appears that the nonadiabatic multiphonon electron-transfer theory can provide the framework for quantitative analysis of reactions involving biological molecules.

Another theory often used to describe electron transfers especially for inorganic complexes is that of Marcus (1963, 1964). The theory in the relative form (Marcus and Sutin, 1974) has been employed to relate self-exchange (k_{11}, K_{22}) and cross-exchange rate constants (k_{12}) of redox processes involving azurin, plastocyanin, stellacyanin, and laccase (Rosenberg et al., 1976; Wherland et al., 1975; Holwerda et al., 1975; McArdle et al., 1974, 1977). From the experimental rate constant for reactions 1 and 3, the self-exchange reaction rate constant can be calculated with the knowledge of the half reduction potentials of the molecules involved. The relative Marcus theory gave a value of $k_{12} \approx 1.6 \times 10^{-4}$ M^{-1} s^{-1}. Since the rate of this reaction was too slow to be measured by ^{1}H NMR, the present study did not provide a test for the relative merits of the different electron-transfer theories. However, most of the redox reactions involving cytochrome molecules cannot be simply correlated with the relative Marcus theory. Wherland and Gray (1976) have computed the self-exchange rate constant for native cytochrome c from the measured rate constants for cross-exchange with Fe(EDTA)$^{2-}$, Co(phen)$_3^{3+}$, Fe(CN)$_6^{3-}$, and Ru(NH$_3$)$_4^{2+}$ and the known self-exchange rate constants of the inorganic redox agents. The computed values ranged from 1.2×10^2 to 3.3×10^7 M^{-1} s^{-1}. These authors also found that corrections for Coulombic contribution or letting the effective active-site radius of cytochrome c be a parameter failed to result in a common value of cytochrome c self-exchange rate constant for the above reactions. In other instances, from the rate of Fe(CN)$_6^{3-}$ oxidation of ^{Fe}cyt c_1 (Yu et al., 1973) and self-exchange rate of Fe(CN)$_6^{3-/4-}$ (Swanson and Ryan, 1973), the relative Marcus theory gave a value of 2.16×10^1 M^{-1} s^{-1}. The poor correlation probably originates from the fact that the absolute Marcus theory is intended for adiabatic processes. The relative Marcus theory is therefore applicable to reactions which are all adiabatic or at least if they are all equally nonadiabatic. There is till now no simple procedure to accommodate reactions which are not equally nonadiabatic in the context of the relative Marcus theory. However, a comparison of this theory with the multiphonon nonadiabatic electron-tunneling theory points a way. In the former the rate constants of cross-exchange and self-exchange reactions are related by a function f,

$$k_{ab}^2 = k_{aa} k_{bb} K_{ab} f \tag{14}$$

where K_{ab} is the equilibrium constant for the cross-exchange process. It can be shown that

$$\log f = \log\left(\frac{|T_{ab}|^2}{|T_{aa}||T_{bb}|}\right) - \log K_{ab} - \log\left(\frac{r_{aa} r_{bb} R_{ab}^2}{r_{ab}^2 R_{aa} R_{bb}}\right) - \frac{1}{2}\log\left(\frac{\Delta_{ab}^2}{\Delta_{aa}\Delta_{bb}}\right) - \frac{1}{4k_B T} \times \left[\frac{2(E_a - E_b)^2 - 4(E_a - E_b)\Delta_{ab} + 2\Delta_{ab}^2 - \Delta_{aa} - \Delta_{bb}}{\Delta_{ab}}\right] \tag{15}$$

In the above equations the Rs are the effective active-site radii and Z is the collision frequency. In the case of self-exchange reactions, $|T_{ab}| \approx |T_{aa}| \approx |T_{bb}|$; $r_{aa} = r_{bb} = r_{ab}$; $\Delta_{aa} \approx \Delta_{bb} \approx \Delta_{ab}$, $f = 1$, $E_a - E_b \ll 1$ eV; and $2(E_a - E_b)^2 \ll 4(E_a - E_b)\Delta_{ab}$, to give

$$\log K_{ab} \approx (E_a - E_b)/k_B T \tag{16}$$

all the reactions are then all equally nonadiabatic, and the two theories are equivalent. When the two electron-transfer partners are not the same, such as one is an inorganic redox agent, or when the self-exchange and cross-exchange reactions are not equally nonadiabatic, then all the other terms in eq 15 need to be considered to obtain the function f which will correctly relate the self-exchange and cross-exchange rate constants.

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